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Acute Postnatal Exposure to Triethyltin in the Rat: Effects on Specific Protein Composition of Subcellular Fractions from Developing and Adult Brain

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16. ABSTRACT

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Acute Postnatal Exposure to Triethyltin in the Rat: Effects on Specific Protein Composition of Subcellular Fractions from Developing and Adult Brain[1]

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ABSTRACT

The morphological maturation of the central nervous system is characterized by ontogenetic changes in proteins associated with specific developmental processes. In this investigation we examined the effects of acute postnatal administration of triethyltin (TET) on the ontogeny of proteins associated with myelinogenesis, synaptogenesis and synaptic junction formation. Myelin, synaptic plasma membrane and synaptosomal cytosolic fractions were prepared from 13-, 22- and 66-day-old Long-Evans rats that had received either saline or TET (3.0 or 6.0 mg/kg i.p.) at 5 days of age. The specific protein composition of each fraction was evaluated by resolution of the fraction proteins on sodium dodecyl sulfate-polyacrylamide slab gels. The presence of marker phosphoproteins associated with each fraction was determined by three criteria: electrophoretic mobility, developmental profile and autoradiography of incorporated phosphate. The specific protein and phosphoprotein content of each fraction was quantified by microdensitometry. The postnatal day 5 administration of TET produced a dose-related decrease in the concentration of myelin basic protein, a myelin-specific marker. This effect was large in magnitude (40–60% of control) and was observed in myelin fractions prepared from 13-, 22- and 66-day-old subjects. No changes in the protein or phosphoprotein content of other subcellular fractions were observed after the day 5 administration of TET. These data indicate that acute postnatal administration of TET may interfere selectively with myelinogenesis.

Recently, both behavioral and neurochemical effects of TET have been assessed in the developing rat. A single exposure to TET on postnatal day 5 results in disruption of developmental behaviors and causes permanent changes in brain weights, behavior and sensorimotor functions (Harry and Tilson, 1981; Reiter *et al.*, 1981). Few neurochemical correlates of this toxicant-induced change in brain and behavior have been examined.

Before the onset of myelination at postnatal day 10 (Norton, 1981), maturation of the rat brain is characterized by rapid oligodendroglia proliferation (Altman, 1970). This process of cellular proliferation has been shown to be particularly vulnerable to toxic insult (Suzuki, 1980). Although cell division during the early postnatal period is primarily glial in nature, there is a considerable degree of postnatal neurogenesis in certain brain areas, *e.g.*, hippocampus and cerebellum (Altman, 1970). Furthermore, the formation of synapses in various areas of the rat brain markedly increases between 1 and 4 weeks postnatally (Aghajanian and Bloom, 1967; Armstrong-James and Johnson, 1970; Crain *et al.*, 1973). Thus, the administration of TET on postnatal day 5 may result in changes in neuronal as well as myelin elements of the CNS. In order to delineate which cellular elements are affected by acute postnatal exposure to TET, we examined the protein composition of three subcellular fractions: M, SPM and SC.

In the nervous system, specific functions are thought to be associated with various cellular and subcellular structures (Bock, 1978). These functions presumably are mediated to a large extent by soluble or membrane-bound proteins that are unique or characteristic to the nervous system. Indeed, a number of nervous system-specific proteins have been identified in the mammalian CNS (for reviews see Zomzely-Neurath and Keller, 1977; Bock, 1978). Thus, nervous system-specific proteins may serve as specific markers of the unique structure and function of the CNS and as such would provide useful indicators of selective neurotoxicity. Therefore, in the present investigation we examined not only the total protein composition of specific subcellular fractions, but extended our observations to include measurements of M basic protein, protein 1 and neurotubulin. These proteins serve as biochemical markers of myelinogenesis, synaptogenesis and synaptic junction formation,

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ABBREVIATIONS: TET, triethyltin; CNS, central nervous system; M, myelin; SPM, synaptic plasma membrane; SC, synaptosomal cytosol; MBP, myelin basic protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels; M_r, molecular weight.

respectively. Because all three marker proteins are phosphorylated by endogenous protein kinases (Steck and Appel, 1974; Lohmann *et al.*, 1978; Burke and DeLorenzo, 1981), we were able to detect and quantify each in SDS-PAGE using protein staining combined with autoradiography of the ^{32}P-labeled protein bands.

Methods

Subjects. Gravid Long-Evans rats (Blue Spruce Farms, Inc., Altamont, NY) were obtained on day 2 of gestation and housed individually in plastic tub cages (45 × 25 × 20 cm) with direct bedding. Pups born between 5:00 P.M. and 5:00 A.M. were considered to be born on the same day (day 0). On day 1, pups were reassigned to litters such that each dam received four male and four female pups and no more than one male and female of her own offspring. At 21 days of age, littermates were weaned and separated by sex and housed four per cage. Animals were maintained in a temperature (22°C ± 2)- and humidity (50% ± 10)-controlled colony room on a 12:12 hr light/dark cycle beginning at 6:00 A.M.

Dosing. Pups were injected (i.p.) on postnatal day 5 with 0.0, 3.0 or 6.0 mg/kg of TET bromide with saline (0.9%) as the vehicle control (10 μl/g b.wt.). All pups in a litter received the same dose and 20 separate litters were used in this study. Subjects were randomly selected from separate litters and sacrificed at 13, 22 or 66 days of age. At each time point no more than one female and one male were selected from each litter.

Preparation of subcellular fractions. After sacrifice by decapitation, brains were removed within 30 sec and immersed in ice-cold 0.32 M sucrose. Brains obtained from control subjects were always prepared in tandem with those obtained from subjects receiving TET. SC, M and SPM subfractions were prepared as described below. Whole brains were homogenized in 10 volumes of 0.32 M sucrose and a crude mitochondrial fraction (P_2) was prepared by differential centrifugation. The P_2 fraction from each treatment group was then subjected to hypoosmotic shock in 500 μl of 5.0 mM Tris HCl (pH 8.0), essentially as described (Jones and Matus, 1974, without sonification, except where noted). The resulting lysate was centrifuged at 140,000 × *g* for 30 min and the supernatant fraction obtained was used as a source of SC (see O'Callaghan *et al.*, 1980, 1982). The membrane fractions recovered during the preparation of the SC fraction were subjected to isopycnic sedimentation on discontinuous sucrose gradients (10, 28.5 and 34%) (w/w) as described (Jones and Matus, 1974). The M-enriched subfraction was harvested from the 10:28.5% interface and an SPM-enriched subfraction was harvested from the 28.5:34% interface. The protein content of each subcellular fraction was assayed according to the method of Bradford (1976) and expressed as a function of brain weight. Bovine plasma γ-globulin was used as a standard. In a given experiment, all protein values for different subcellular fractions (regardless of treatment group) were equalized before electrophoresis.

Resolution of subcellular fraction proteins by SDS-PAGE. The SC, M and SPM proteins were resolved on SDS-polyacrylamide vertical slab gels (10 × 16 × 0.15 cm) (Hoefer Scientific Instruments, Inc., San Francisco, CA). Routinely, a 50-μl aliquot containing 17 μg of fraction protein was applied to each of the 20 wells in a slab. Except where noted, the acrylamide concentration was 6 and 10% in the stacking and resolving gels, respectively (36.5:1, acrylamide:bisacrylamide). The stacking, resolving and running gel buffers were those described by Laemmli (1970). The samples were subjected to electrophoresis under conditions of constant power (10 w/gel) at 18–20°C until the tracking dye reached the bottom of the gel (3.5–4.0 hr). After electrophoresis, the gels were fixed and stained for protein overnight with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid. Gels were destained by diffusion in 30% methanol and 10% acetic acid and dried under heat and partial vacuum. Autoradiographs of the ^{32}P-labeled proteins (see below) were obtained by placing the dried gels in close contact with Kodak RP X-ray film for a period of up to 24 hr. Short exposure times were achieved by using intensifying screens (Dupont Cronex Lightning-Plus, CGR Corp., Raleigh, NC). The apparent M_rs of the resolved proteins were determined from M_r standards that had been subjected to electrophoresis under conditions identical to the tissue samples.

Detection of marker proteins. Specific marker proteins were located in the dried gels and corresponding autoradiographs on the basis of electrophoretic mobility, pattern of incorporated phosphate and developmental profile. MBP, a metabolically stable protein (Sabri *et al.*, 1974; Fischer and Morell, 1974; Shapira *et al.*, 1981) unique to M and glial cell lines that produce M (Shapira *et al.*, 1978), was chosen as a marker of the M fraction. The synapse-associated, neuron-specific protein, protein 1 (for a review, see Greengard, 1979), was utilized as a marker of the SPM fraction. Tubulin, a major cytoplasmic protein in brain that plays a role in axoplasmic transport, axonal elongation and synapse formation (see Shelanski and Feit, 1972), was employed as a marker of SC. The M_r [Norton, 1981 (MBP); Ueda and Greengard, 1977 (protein 1); Blitz and Fine, 1974 (tubulin)], endogenous phosphorylation characteristics [Steck and Appel, 1974 (MBP); Huttner and Greengard, 1979 (protein 1); Burke and DeLorenzo, 1981 (tubulin)] and ontogenetic profile [Banik and Smith, 1977 (MBP); Lohmann *et al.*, 1978 (protein 1); Schmitt *et al.*, 1977 (tubulin)] have been determined by SDS-PAGE for all three marker proteins. The specific marker proteins were detected in the stained gels and corresponding autoradiographs. Both MBP and tubulin were identified in the stained gels on the basis of electrophoretic mobility (M_r) and ontogenetic profile. Protein 1 could not be readily identified in stained gels. Autoradiographic detection of all three markers was achieved by phosphorylating the subcellular fractions, *in vitro*, before electrophoresis. The standard phosphorylation assay contained: 100 μg of the SC, M or SPM proteins; 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (pH 6.5); 10 mM $MgCl_2$; and 5 μM ATP containing [γ-^{32}P]ATP (1×10^7 dpm/nmol). The SC proteins were incubated in the presence of 50 μM $CaCl_2$. The SPM subfraction was incubated in the presence of 10 μM cyclic AMP. Assays were incubated at 30°C in a final volume of 200 μl. The phosphorylation reaction was initiated by the addition of a 25-μl aliquot of the SC, M or SPM subfraction (suspended in 5.0 mM Tris HCl, pH 8.0). The reaction was terminated after 1 min (SC) or 15 sec (M and SPM) by the addition of 100 μl of an SDS-PAGE sample buffer containing 0.125 M Tris HCl (pH 6.8), 4% SDS, 20% glycerol and 10% 2-mercaptoethanol. All samples were then heated in a water bath for 2 min at 90°C before electrophoresis.

Quantification of marker proteins. The amount of each marker protein resolved by SDS-PAGE was measured by microdensitometry of the stained gels and corresponding autoradiographs using a Quick-Scan (Helena Laboratories, Beaumont, TX) transmission microdensitometer. The amount of protein (stained bands) or phosphoprotein (autoradiographic bands) was quantified from the densitometric scans by determining the areas under the peaks corresponding to specific protein or phosphoprotein bands. A linear relationship was observed between microdensitometry values and the full range of observed staining intensities or autoradiographic exposures. The values obtained for the two peaks corresponding to the large MBP were combined as were those corresponding to α- and β-tubulin. Values for the small basic protein were not determined. Densitometric values for protein 1 were limited to those obtained from the autoradiographs. The background color of the dried gels or the background darkness of the corresponding autoradiographs was taken as the base-line reading for densitometric determinations. The data are expressed as a percentage of total protein or as a percentage of control and values obtained from males and females were combined. Comparisons between control and experimental groups were only made between samples that had been subjected to electrophoresis on the same gel. Statistical comparisons were based on Student's *t* test for group observations.

Materials. [γ-^{32}P]ATP (10–40 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Piperazine-N,N′-bis(2-ethanesulfonic acid) and Tris were obtained from Calbiochem (San Diego, CA). Sucrose (grade II) was from Sigma Chemical Co. (St. Louis, MO). All other materials used in the preparation of the polyacrylamide gels and associated buffers were of electrophoresis grade and were purchased

from Bio-Rad Laboratories (Richmond, CA). TET bromide was purchased from Alfa Products (Danvers, MA).

Results

Brain weights and subcellular fraction protein yields. Administration of TET on postnatal day 5 resulted in a significant decrease in brain weights at 13, 22 and 66 days of age (table 1). Total protein yields for each subcellular fraction were also obtained at these ages (table 1). M, but not SC or SPM, proteins were significantly reduced at 22 days of age as a consequence of TET administration (6.0 mg/kg) on postnatal day 5. At 13 or 66 days of age, the protein yields for all three subcellular fractions did not differ between the control and TET groups. The body weights of the TET-treated rats did not differ significantly from controls at either 22 or 66 days of age; however, at 13 days of age the body weights of rats that had received 6.0 mg/kg of TET were reduced by 13% ($P < .01$) (data not shown). Sex-related differences were not observed for any of the measurements employed in this investigation.

Electrophoretic profiles and autoradiographs of subcellular fraction proteins. The electrophoretic profiles and corresponding autoradiographs of SC, M and SPM proteins were obtained from control and TET-treated subjects at 13, 22 and 66 days of age. A representative series of protein staining patterns and corresponding autoradiographs obtained from 22-day-old animals is presented in figure 1. SDS-PAGE of SC, M or SPM proteins resulted in the resolution of at least 50 Coomassie blue stained bands (M_r, 10,000–200,000) in each fraction. With the exception of protein 1, protein bands corresponding to the specific marker proteins associated with each subcellular fraction could be identified in both the staining and autoradiographic profiles. A band corresponding to the neuron specific protein, protein 1, could only be detected in the SPM fraction with the aid of autoradiography. Qualitative and quantitative analysis of the protein composition of the SC or SPM fractions did not reveal any differences between the control or TET treatment groups. In contrast, a reduction in specific M fraction proteins was observed in both the staining and autoradiographic profiles obtained from subjects that had received 6.0 mg/kg of TET on day 5. This effect was not limited to the M marker protein, the phosphorylated MBP doublet (M_r, 15,000–17,000),

TABLE 1

Effect of acute postnatal administration of TET on protein yields obtained from brain subcellular fractions

Each value represents the mean ± S.E.M. of six independent observations.

Age	N	Dose of TET	Brain Wts.	SC	M	SPM
days		mg/kg	g		mg protein/g brain	
13	6	0.0	1.36 (±0.02)	1.33 (±0.18)	1.40 (±0.15)	2.45 (±0.22)
	6	3.0	1.25 (±0.02)**	1.36 (±0.16)	1.36 (±0.10)	2.17 (±0.20)
	6	6.0	1.06 (±0.05)**	1.78 (±0.15)	1.25 (±0.10)	2.40 (±0.25)
22	6	0.0	1.59 (±0.02)	3.50 (±0.14)	2.21 (±0.25)	3.17 (±0.41)
	6	3.0	1.49 (±0.02)**	3.72 (±0.16)	2.18 (±0.28)	3.19 (±0.42)
	6	6.0	1.33 (±0.02)**	3.55 (±0.11)	1.65 (±0.19)*	2.96 (±0.39)
66	6	0.0	1.98 (±0.04)	2.44 (±0.06)	3.36 (±0.25)	2.60 (±0.18)
	6	3.0	1.90 (±0.03)	2.47 (±0.12)	3.36 (±0.23)	2.55 (±0.19)
	6	6.0	1.55 (±0.02)**	2.41 (±0.11)	2.90 (±0.18)	2.82 (±0.25)

* Significantly different from control, $P < .05$; ** significantly different from control, $P < .01$.

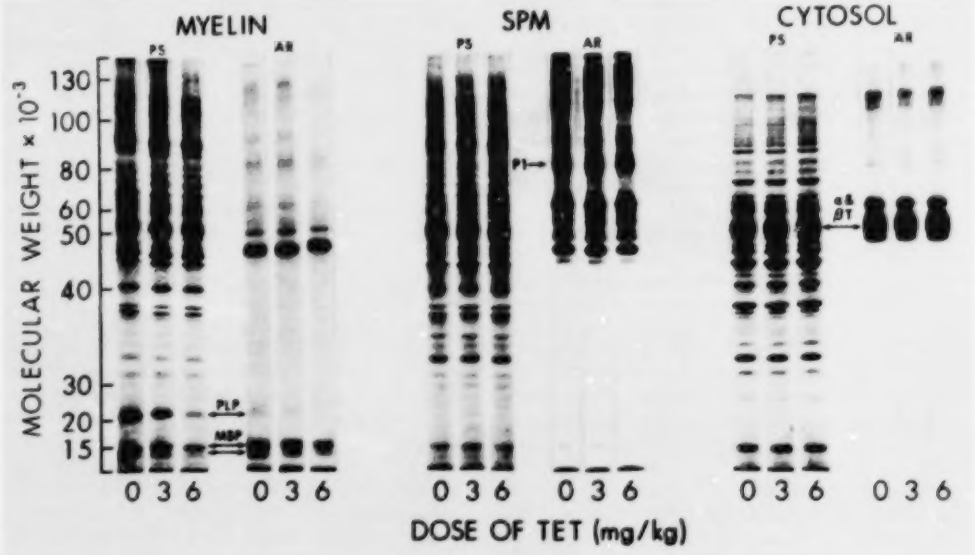

Fig. 1. Protein staining (PS) pattern and corresponding autoradiographs (AR) of M, SPM and synaptosomal cytosolic (cytosol) fractions from 22-day-old male rats. Fractions were obtained from rats treated on day 5 with saline or TET. Proteins were stained with Coomassie Blue R-250. Proteins or phosphoproteins with electrophoretic mobilities corresponding to proteolipid protein (PLP), large MBP, protein 1 (P_1) and α- and β-tublin (α and βT) are indicated by arrows. See "Methods" for criteria used to identify specific proteins.

but was also observed for a protein of apparent M_r of 22,000. The ontogenetic profile (day 13, 22 and 66) and electrophoretic mobility of this protein (Banik and Smith, 1977; Jones and Matus, 1975; Burgoyne *et al.*, 1981) as well as its failure to incorporate phosphate *in vitro* (Steck and Appel, 1974) identify it as the M-associated proteolipid protein. Although MBP and proteolipid protein were both consistently affected by postnatal administration of TET, only MBP is significantly phosphorylated; therefore, quantitative data were obtained only for this protein. Furthermore, as the small MBP was not highly phosphorylated on postnatal day 13, MBP determinations at all time points were restricted to the large basic protein component.

M fractions prepared as described under "Methods," without sonification, are known to be contaminated with SPM-derived fragments (Jones and Matus, 1974). It is apparent (fig. 1) that several protein staining bands in the M fraction exhibit electrophoretic mobilities that are similar to major bands found in the SPM fraction. Because contamination of the M fraction with SPM-derived proteins may have contributed to the results observed in figure 1, we subjected the homogenates from several experiments to sonification before density gradient centrifugation. The resulting M fraction proteins were then resolved in 15% gels. Typical electrophoretic profiles of M proteins prepared in this fashion (from 22-day-old subjects) are presented in figure 2. The proteolipid protein, large basic protein doublet and small basic protein compose the major M proteins in the sonified preparation (fig. 2). In agreement with the results presented in figure 1, TET produced a dose-related decrease in the proteolipid and large MBP. Additionally, the small MBP was also decreased by TET administration on postnatal day 5. The densely staining protein of M_r 55,000 may be the M specific Wolfgram protein (fig. 2). It was not affected by TET administration.

Quantification of marker proteins. The administration of TET on postnatal day 5 did not affect the microdensitometry values for neurotubulin or protein 1 obtained from 13-, 22- or 66-day-old subjects (data not shown). Microdensitometry values obtained from the staining profiles of MBP are shown in figure 3. As has been reported (Jones and Matus, 1975; Burgoyne *et al.*, 1981), the MBP content of M subfractions could not be detected at 13 days of age. Therefore, quantitative data based on protein staining was limited to values obtained from the 22- and 66-day-old subjects. Postnatal day 5 administration of TET resulted in a dose-related decrease in MBP at both 22 and 66 days of age. This decrease was greatest (50% of control) at 22 days but was still significantly reduced at 66 days. After phosphorylation, *in vitro*, MBP was detected by autoradiography and quantified by microdensitometry. These data are shown in figure 4. In agreement with previous work (Steck and Appel, 1974), we found that endogenously phosphorylated MBP could be detected and quantified during the early postnatal period. Postnatal TET administration caused a dose-related decrease in the phosphorylated MBP at 13, as well as 22 and 66 days of age. These data extend our observations based on protein staining to 13-day-old animals, a point in postnatal development that coincides with the earliest stages of myelinogenesis (Norton, 1981).

Discussion

We have demonstrated that exposure to TET on postnatal day 5 in the rat results in a persistent decrease in total M protein and in the M marker, MBP. The total and specific protein content of other CNS subcellular fractions were not affected, suggesting that the effects of TET were specific to the M. Previous investigators have also found deficits in myelination after acute (Wender *et al.*, 1974) or chronic (Blaker *et al.*, 1981) administration of TET to the rat during the early postnatal period.

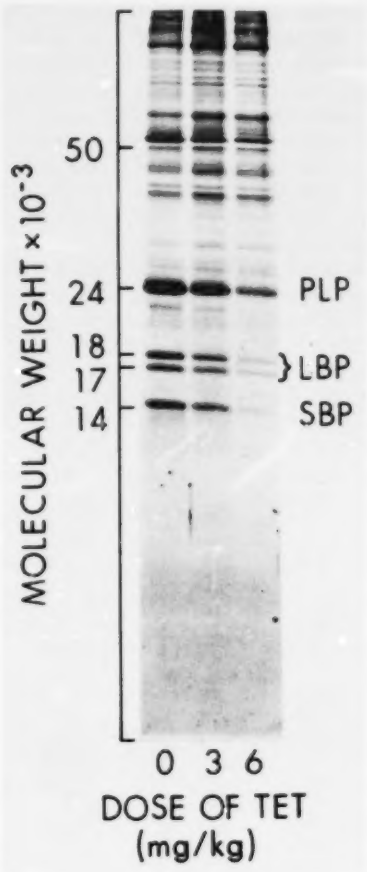

Fig. 2. Protein staining pattern of sonified M fractions obtained from 22-day-old female rats. Fractions were obtained from rats treated on day 5 with saline or TET. The acrylamide concentration was 6 and 15% in the stacking and resolving gels, respectively (36.5:1, acrylamide-bisacrylamide). Proteins were stained with Coomassie Blue R-250. Abbreviations are as follows: proteolipid protein, PLP; large myelin basic protein, LBP; small myelin basic protein, SBP.

MBP, protein 1 and neurotubulin were chosen as specific markers of the M, SPM and SC fractions, as their ontogeny coincides with three critical developmental processes in the CNS: myelinogenesis (MBP) (Banik and Smith, 1977; Jones and Matus, 1975; Burgoyne *et al.*, 1981), synaptogenesis (protein 1) (Lohmann *et al.*, 1978) and the formation of synaptic junctional complexes (tubulin) (Shelanski and Feit, 1972; Jones and Matus, 1975). The use of SDS-PAGE to examine polypeptide components of each subcellular fraction and to detect and quantify nervous system-specific marker proteins has been used previously [*e.g.*, see Barbarese *et al.*, 1978; Reigner *et al.*, 1981 (MBP); Lohmann *et al.*, 1978 (protein 1); Schmitt *et al.*, 1977 (tubulin)] and proved to be the most sensitive indicator of the effects of acute postnatal exposure to TET. Several proteins may comigrate in SDS gels with the proteins employed as markers for the specific subcellular fractions. The use of phos-

phorylated markers with known ontogenetic profiles makes the positive identification of these proteins more likely. However, identification of the specific marker proteins will require resolution by two-dimensional gel techniques or the use of specific radioimmunoassays (Cohen *et al.*, 1980; Goelz *et al.*, 1981).

Our results indicated that TET only affected the M fraction and, within this fraction, only the M-specific proteins were altered. These included both the proteolipid protein (see figs. 1 and 2) as well as the large and small MBPs. Quantification of the large MBP by microdensitometry of the autoradiographs or of the stained gels revealed a persistent 40 to 60% reduction in this marker of myelinogenesis. The content of MBP in most areas of the CNS of the rat reaches a peak by postnatal day 40 (Zgorzalewicz *et al.*, 1974; Banik and Smith, 1977; Day, 1981) and stabilizes at that level well into adulthood (Zgorzalewicz *et al.*, 1974; Banik and Smith, 1977). MBP that accumulates during development remains essentially stable (Shapira *et al.*, 1981), a finding that supports the hypothesis that MBP may play a role in stabilizing the compaction of M lamellae (Smith, 1977). In light of these reports, the deficits in MBP as a consequence of TET administration on postnatal day 5 are indicative of a permanent deficit in myelination.

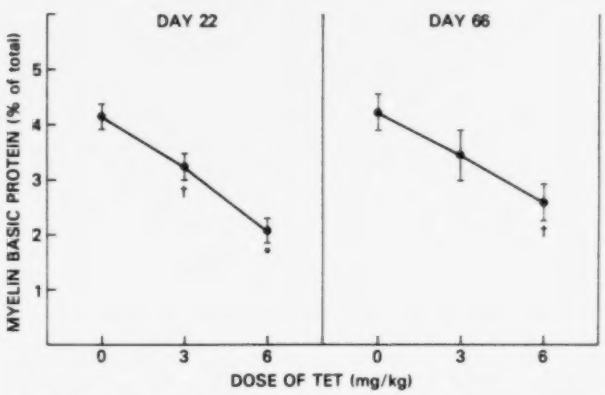

Fig. 3. The effects of TET on the concentration of MBP. Values were obtained from M fractions prepared from 22- and 66-day-old rats that had received TET on day 5. Each point represents the mean + S.E.M. of at least six independent observations. †Significantly different from the control, $P < .05$; * significantly different from control, $P < .01$.

We suggest that the reduction in MBP on days 13, 22 and 66 is a consequence of a TET-induced alteration of the proliferating oligodendroglia. Such an effect could be due to a cytotoxic action of TET or the result of a depression of early M synthesis. A general cytotoxic action of TET on day 5 is unlikely since the SPM and SC fractions were not affected. However, a general metabolic insult on day 5 would be expected to affect the rapidly proliferating oligodendrogliocyte population and may account for the results obtained in the present investigation. That TET is a general metabolic inhibitor has been well documented by studies demonstrating its effects on mitochondrial function, *in vitro* (Stockdale *et al.*, 1970). Additionally, it is possible that the effect of TET on day 5 is mediated through a direct action on MBP. On postnatal day 5, MBP is present in high concentration in M-forming oligodendroglia (Sternberger *et al.*, 1978) and may play an important role in the initial development of the M sheath (Sternberger *et al.*, 1978).

Although TET did not affect the protein yield, composition or specific marker content of the SPM or SC fractions, an action of TET on these non-M components of the CNS cannot be ruled out. Whereas MBP is metabolically stable (Fisher and Morell, 1974; Sabri *et al.*, 1974; Shapira *et al.*, 1981), most proteins in the developing brain are subject to turnover (Lajtha and Dunlop, 1981). As only steady-state levels of specific proteins were examined, the possibility exists that TET may influence the dynamic state of proteins present in cell types and subcellular organelles that are both M and non-M in origin. It is also possible that the effects of TET may be restricted to an action on proliferating cell populations. Because extensive postnatal neurogenesis is largely restricted to the hippocampus and cerebellum (Altman, 1970), an effect of TET on these structures may have been masked by an examination of the whole brain.

We have employed endogenous phosphorylation reactions to quantify as well as identify three marker proteins in heterogeneous mixtures of proteins resolved from subcellular fractions of whole brain. Because protein 1 could not be identified in protein staining profiles of SPM fractions, it was routinely detected by endogenous phosphorylation (see Greengard, 1979). Quantification of protein 1 in particulate fractions of developing

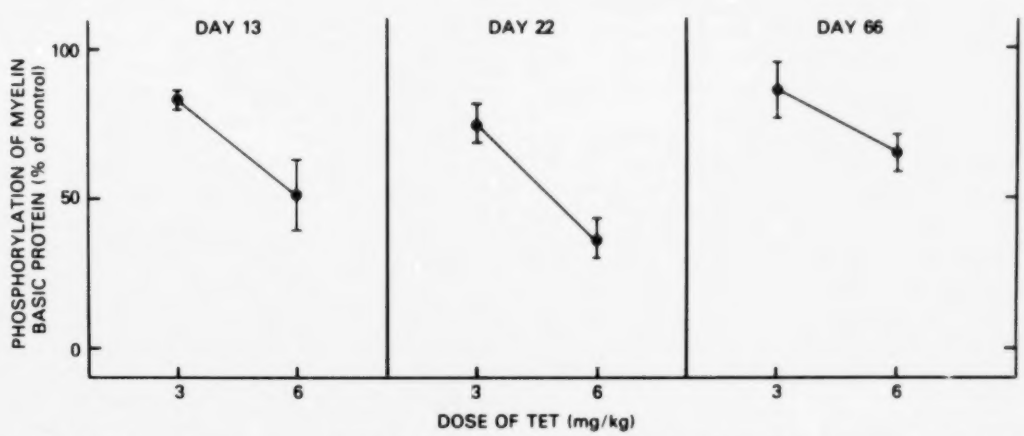

Fig. 4. The effects of TET on the amount of phosphomyelin basic protein. The net incorporation of phosphate into protein was quantified from the densitometric scans of the autoradiographs and is expressed as a percentage of control phosphorylation. Each point represents the mean + S.E.M. of at least six independent observations.

brain was also achieved by endogenous phosphorylation, as the amount of protein 1 assayed in this fashion is indistinguishable from that obtained for the extracted and purified protein (Lohmann *et al.*, 1978). Although MBP and tubulin were detected and quantified by dye-binding, they were also analyzed by protein phosphorylation. Whereas endogenous phosphorylation assays can indicate the presence of a specific protein, the net incorporation of phosphate into a given protein may be influenced by a number of factors other than the amount of endogenous substrate present (see Bär *et al.*, 1981, for a discussion). Our data indicated that TET caused a dose-related decrease in phosphomyelin basic protein without affecting protein 1 or phosphoproteins that comigrate with α- and β-tubulin. The relative decrease in MBP assessed by endogenous phosphorylation was roughly equivalent to that observed by dye-binding (see figs. 3 and 4). Thus, it appears that although several factors may affect the net incorporation of phosphate into the marker proteins employed in this study, these and possibly other phosphoproteins may be detected and measured after phosphorylation by endogenous protein kinases.

Several authors have suggested that the observed toxicity of the heavy metals may be in part due to heavy metal-induced deficiencies in essential trace elements such as copper and zinc (Mahaffey, 1980). For example, Klauder and Petering (1977) have noted the similarities between lead and copper deficiency-induced anemias. Interestingly, anomalies in M formation have been linked to deficiencies in copper during both the prenatal and neonatal periods (Everson *et al.*, 1968; DiPaolo *et al.*, 1974; Zimmerman *et al.*, 1976). Copper deficiency is also known to produce depigmentation as it is an essential component of tyrosinase which participates in the conversion of tyrosine to melanin (Malmstrom and Ryden, 1968). In the present study, pigmented coat areas of the TET-treated rats showed a dose-related decrease in pigmentation and these areas were "grayish" in coloration. These effects gradually dissipated and were not present after approximately 50 days of age. We are currently investigating the possibility that some of the effects of TET observed in the present study may be related to a copper deficiency.

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